

IL-25 Promotes Th2 Immunity Responses in Airway Inflammation of Asthmatic Mice via Activation of Dendritic Cells

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Abstract—Allergic asthma occurs as a consequence of inappropriate immunologic inflammation to allergens and characterized by Th2 adaptive immune response. Recent studies indicated that interleukin (IL)-25, a member of the IL-17 cytokine family, had been implicated in inducing Th2 cell-dependent inflammation in airway epithelium and IL-25-deficient mice exhibit impaired Th2 immunity responses; however, how these cytokines influence innate immune responses remains poorly understood. In this study, we used ovalbumin (OVA) sensitization and challenge to induce the murine asthmatic model and confirmed by histological analysis of lung tissues and serum levels of total and OVA-specific immunoglobulin (Ig)-E. The expression of IL-25 was detected by quantitative real-time PCR and immunohistochemistry, respectively, and the dendritic cells (DCs) activation was detected by levels of CD80 and CD86 in bronchoalveolar lavage fluid (BALF) by flow cytometry. The mice sensitized and challenged with OVA showed high expression of IL-25 in both mRNA and protein levels in lungs. We detected the expression of CD80 and CD86 in BALF was also increased. A tight correlation between IL-25 mRNA and other Th2 cells producing cytokines such as IL-4, IL-5, and IL-13 in BALF was identified. Furthermore, when the asthmatic mice were treated with inhaled corticosteroids, the inflammatory cells infiltration and the inflammatory cytokines secretion were significantly decreased. In this study, we show that IL-25 promoted the accumulation of co-stimulatory molecules of CD80 and CD86 on DCs and then induced the differentiation of prime naive CD4⁺ T cells to become proinflammatory Th2 cells and promoted Th2 cytokine responses in OVA-induced airway inflammation. The ability of IL-25 to promote the activation and differentiation of DCs population was identified as a link between the IL-17 cytokine family and the innate immune response and suggested a previously unrecognized innate immune pathway that promotes Th2 cytokine responses in asthmatic airway inflammation. Inhaled corticosteroids might be capable of inhibiting the promotion of IL-25 and present a promising strategy for the treatment of asthma

KEY WORDS: interleukin-25; asthma; airway inflammation; Th2 immunity Responses; dendritic cells.

INTRODUCTION

Allergic asthma is a chronic airway inflammatory disease associated with a predominant Th2 immunity response to allergens, leading to eosinophils and mast cells

infiltration in airway, goblet cell hyperplasia, and airway hyperreactivity (AHR) [1]. Interleukin (IL)-25 (IL-17E) is a member of family of six proteins labeled IL-17A–F and appears to be involved primarily in the promotion of Th2 immunity responses whereas other members of the IL-17 family have biological activities similar to Th1 inflammatory cytokines [2]. Recent studies have established that high level of IL-25 was associated with airway inflammatory disease such as asthma in humans and mice [3]. But how these cytokines influence the innate immune responses and adaptive immune responses remains poorly defined. Here, we hypothesize that IL-25 expressed in

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airway epithelium could promote the activation of dendritic cells (DCs) and induced activated DCs to prime naive CD4⁺ T cells to differentiate into Th2 cells and promoted Th2 cytokine responses. IL-25, the IL-17 cytokine family, acted as the link between the innate immune response and the adaptive proinflammatory Th2 cytokine responses in asthmatic airway inflammation. To evaluate the role of IL-25 in the development of asthma, we generated the murine asthmatic model with ovalbumin (OVA) and identified the expression of IL-25 and Th2-type inflammation in bronchoalveolar lavage fluid (BALF) in this process. We found that the expression of IL-25 mRNA and protein was significantly elevated in epithelial cells and the expression of CD80 and CD86 in BALF was also increased on DCs of asthmatic mice, whereas inhaled corticosteroids (ICSs) decreased the inflammatory cells infiltration and inflammatory cytokines secretion. Tight correlation between IL-25 mRNA and other Th2 cells producing cytokines such as IL-4, IL-5, and IL-13 in BALF was identified [4]. This finding showed that IL-25 maybe involved in a range of asthmatic mediators secretion and might play a crucial role in activation and differentiation of DCs population and then maintaining Th2 central memory and sustaining asthmatic inflammation while ICSs was capable of inhibiting the positive feedback amplifying loop.

MATERIALS AND METHODS

Animals

We obtained BALB/c mice ($n=30$, weight 25 ± 5 g, female) from the Animal Centre of Shandong University, Shandong, China. All animal experiments undertaken in this study were done so with the approval of the Institutional Animal Care and Use Committee [5] of Shandong University. The mice were randomly divided into three groups — asthma group ($n=10$), control group ($n=10$) and budesonide-treated group ($n=10$).

Model Generation

Mice in the asthma group were injected intraperitoneally with 100 μ g OVA in 1 mg aluminum hydroxide (Sigma) in a total volume of 0.25 ml of PBS twice at days 1 and 8, and then challenged with OVA aerosols (1 % in PBS) for 30 min per day for 7 consecutive days. The control mice were sensitized and challenged with PBS instead. For the budesonide-treated experiment, mice were given inhaled budesonide suspension (0.5 mg per mouse; R&D) 30 min before each challenge [6].

Bronchoalveolar Lavage (BAL) and Serum Collected

The mice were deeply anesthetized by an intraperitoneal injection of chloral hydrate (4 mg/g body weight) at 24 h after the last challenge. Blood was collected by cardiac puncture and serum was stored at -70°C after centrifugation [7]. Collection of BALF was performed with PBS lavage six times (0.5 ml each time) through the cannulated trachea. A total of 2.5 ml BAL fluid was collected from every mouse [8]. The supernatant was decanted and stored at -80°C for further analysis.

Histology

Lungs were excised completely from the chest cavity, and fixed with 10 % neutral buffered formalin. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) [9]. All images were developed at $\times 40$. The remaining lungs were stored at -80°C . Total RNA and protein were extracted to perform polymerase chain reaction (PCR) and immunohistochemistry analysis, respectively.

Analysis of Inflammatory Cytokines in BALF and IgE in Serum

We examined the cellular influx in the BALF by cell counting plate. BAL samples were centrifuged and the cell pellet was subjected to count the number of eosinophils with Wright's staining. The levels of IgE in serum and IL-25, IL-4, IL-5, IL-13 and interferon (IFN)- γ in the BAL supernatants were examined by enzyme linked immunosorbent assay (ELISA) kits (R&D Systems).

Quantitative Real-Time PCR

Total RNA was isolated from mouse bronchus by the RNeasy Mini Kit (Qiagen, West Sussex, UK) and then reverse transcribed with random hexamers and AMV reverse transcriptase (Promega, Southampton, UK). cDNA was amplified by SYBR Green PCR Master Mix Reagent (Qiagen) in Rotor Gene 3000 (Corbett Research, Sydney, Australia). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Primers used were as follows: GAPDH (131 bp) forward: 5'-AACAGGCGTCCCTTTCCGA-3', reverse: 5'-GCCAAGATGCCCTTCAGT-3', IL-25 (192 bp) forward: 5'-CAGCAAAGAGCAAGAACC-3', reverse: 5'-CCCTGTCCAACATAGC-3'. The quantity of IL-25 expression was assessed with the comparative Ct method.

Table 1. Concentration of Total and OVA-Specific IgE in Serum of Mice

Group	<i>n</i>	Total IgE (ng/ml)	OVA-IgE (EU/ml)
Asthma	10	216±31 ^a	44±5 ^a
Control	10	29±3	<10

Blood was collected by cardiac puncture and detected the IgE in serum after centrifugation

Immunohistochemistry Analysis

The sections were dewaxed and rehydrated, and antigen retrieval was performed with 10 mM sodium citrate (pH 6.1). The slides were incubated with an anti-IL-25 antibody (Santa Cruz Biotechnology, USA) (1:25) overnight at 4 °C and then processed with polyclonal rabbit anti-goat immunoglobulins/horseradish peroxidase (HRP; 1:100) for 30 min at 37 °C. The nuclei were counterstained with hematoxylin. Control slides were incubated with normal Ig instead of a primary anti-body. IL-25-positive areas were quantified by densitometry using the image-pro plus software (Media Cybernetics, USA).

Flow Cytometry

BAL samples were centrifuged and the cell pellet was subjected to red blood cell (RBC) lysis. BAL single-cell suspensions were kept in fluorescence-activated cell sorting (FACS) EDTA buffer (PBS, 0.5 % bovine serum albumin, 5 mM EDTA, 0.1 % azide) until immunofluorescent labeling. Single-cell suspensions were preincubated with Fc receptor-blocking antibody (anti-CD16/CD32, clone 2.4G2) to reduce nonspecific binding. Biotinylated anti-CD11c (N418; BD Bioscience) monoclonal antibody was

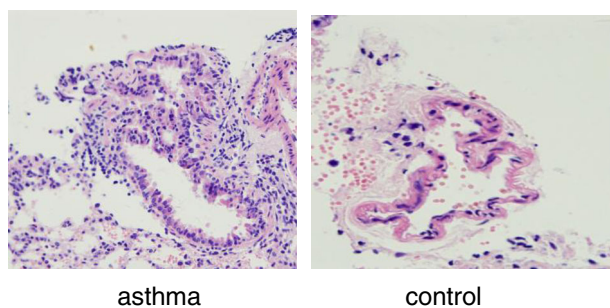


Fig. 1. Lung histology. Pathological sections of lung tissues were harvested 24 h after last challenge, fixed with buffered formalin, and 5-μm sections were analyzed by HE staining. Original magnification, ×40.

Table 2. Inflammatory Responses in the Airway

Group	<i>N</i>	Total cell count (×10 ⁴ /ml)	EOS	
			Absolute count (×10 ⁴ /ml)	Percentage (%)
Asthma	10	52.18±23.65 ^a	22.38±13.06 ^a	4.41±2.93
Control	10	21.6±3.01	1.24±0.79	4.07±1.26

Analysis of BALF total cell number and classified inflammatory cells of BALB/c mice by cell counting plate. Bars depict the mean ± SD

^a Indicates a *p* value of less than 0.05, compared with control group

used to identify mouse DCs. Cells were washed twice with FACS buffer and then analyzed.

Statistical Analysis

All data are demonstrated as mean ± standard deviation (SD). Both the levels of cytokines and the quantity of IL-25 were analyzed by one-way analysis of variance. All test results were considered significant at *p*<0.05.

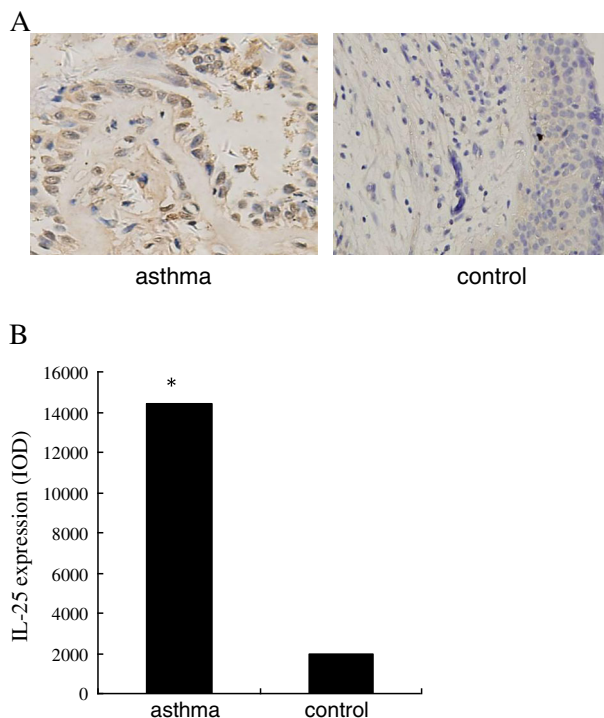


Fig. 2. The expression of IL-25 protein in lung by immunohistochemistry analysis. The tracheal explants of mice were extracted to perform immunohistochemistry analysis. IL-25-positive areas were quantified by densitometry using the image-pro plus software.

Table 3. The Expression of IL-25, IL-4, IL-5, IL-13 and IFN- γ in BALF by ELISA

Group	N	IFN- γ	IL-4	IL-5	IL-13	IL-25
Asthma	10	70.96 $\pm$ 2.08 ^a	58.11 $\pm$ 8.09 ^a	97.41 $\pm$ 11.04 ^a	81.73 $\pm$ 8.25 ^a	189.67 $\pm$ 44.15 ^a
Control	10	27.58 $\pm$ 3.40	20.30 $\pm$ 1.88	28.17 $\pm$ 4.01	26.53 $\pm$ 1.09	70.96 $\pm$ 19.31
<i>F</i>		116.12	231.04	85.04	79.87	103.08
<i>P</i>		<0.01	<0.01	<0.01	<0.01	<0.01

Analysis of inflammatory cytokines in BALF by ELISA. Results are expressed as mean $\pm$ SD

RESULTS

Asthmatic Murine Model

The murine asthmatic model was established with OVA sensitization and challenge. This model was confirmed by detecting airway inflammation, serum level of IgE and lung histopathology. As shown in Table 1, the serum level of IgE was increased dramatically in OVA-challenged mice relative to the value in the control group (Table 1). Histological analysis demonstrated an asthmatic phenotype with significantly increased airway eosinophil infiltration, goblet cell metaplasia and airway hyperresponsiveness (Fig. 1). All these data indicate that we successfully generated the asthma mouse model by OVA sensitization and challenge.

Inflammatory Cells

To determine the inflammatory response of mice, we examined the cellular influx in the BALF by cell counting plate (Table 2). The number of total BALF cells was much higher in OVA group than in the control group (Table 2). Mice exposed to OVA also demonstrated a significant increase in the number of eosinophils and neutrophils (Table 2) as well as goblet cell metaplasia and airway mucus secretion in histological analysis (Fig. 2) compared with the control group.

Inflammatory Cytokines

Mice exposed to OVA displayed more significant AHR and recruitment of inflammatory cells such as eosinophils (Fig. 1). In the supernatants of BALF, we also detected much higher secretion of IL-4, IL-5, IL-13 and IL-25 while lower level of IFN- γ than the control group (Table 3). These results indicate that IL-25 worked in synergy with other Th2-type cytokines such as IL-4, IL-5 and IL-13 to induce the recruitment of eosinophils in OVA induced asthmatic airway inflammation and thus promoted the Th2-type inflammatory responses in asthma.

Both mRNA and Protein of IL-25 Expressed in OVA-Induced Mice

To identify the role of IL-25 in OVA-induced asthma mice, we detected the expression of IL-25 mRNA by quantitative real-time PCR and IL-25 protein by immunohistochemistry, respectively. The expression of IL-25 mRNA and protein on tracheal of mice exposed to OVA were significantly higher than that of mice in control group (Table 4, Fig. 2). Mice exposed to OVA showed more significant airway inflammation characterized by higher amounts of eosinophils and neutrophils infiltration as well as the high secretion of cytokines IL-4, IL-5, IL-13 and IL-25 in BAL supernatants (Table 3).

Co-stimulators Expressed on DCs in OVA-Induced Asthmatic Mice

To investigate the role of IL-25 in promoting innate immunity responses in asthmatic airway inflammation, the levels of CD80 and CD86 on DCs were detected by flow cytometry. The results were expressed as mean $\pm$ SD. We found that the levels of CD80 (90.59 $\pm$ 6.23 %) and CD86 (92.38 $\pm$ 5.06 %) on DCs were significant up-regulated in BALF of OVA-induced asthmatic mice compared with the control group (Fig. 3).

Table 4. Quantitative Detection of IL-25 mRNA in Airway Epithelium

Group	N	GAPDH -Ct (Ct-G)	IL-25-Ct (Ct-25)	2 ^{-$\Delta\Delta$Ct}
asthma	10	15.79 $\pm$ 0.81	20.09 $\pm$ 5.87	1.38 $\pm$ 0.07 ^a
control	10	14.02 $\pm$ 0.57	16.65 $\pm$ 0.74	0.97 $\pm$ 0.03
<i>F</i>		42.75	59.82	105.34
<i>P</i>		<0.01	<0.01	<0.01

The expression of IL-25 mRNA in airway epithelium was detected by comparative Ct value. Results are expressed as mean $\pm$ SD

^a Indicates a *p* value of less than 0.05, compared with control group

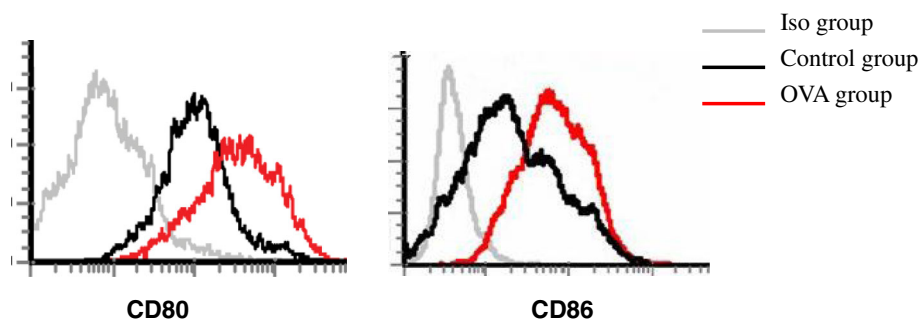


Fig. 3. The expression of CD80 and CD86 on DCs were detected by flow cytometry.

Tight Connection between IL-25 and Inflammatory Cytokines

To clarify the effect of IL-25 on Th2-type cytokines, we did a co-relational analysis between IL-25 mRNA and IL-4, IL-5, IL-13 and IFN- γ in BALF. We found that the

expression of IL-25 mRNA was tightly positive correlated with the levels of IL-4 ($r=0.748$), IL-5 ($r=0.691$), and IL-13 ($r=0.793$) and negative correlated with the levels of IFN- γ ($r=-0.574$) (Fig. 4). Thus Th2-type inflammation played a pivotal role in maintaining and sustaining asthmatic inflammation, and we presumed that IL-25 could

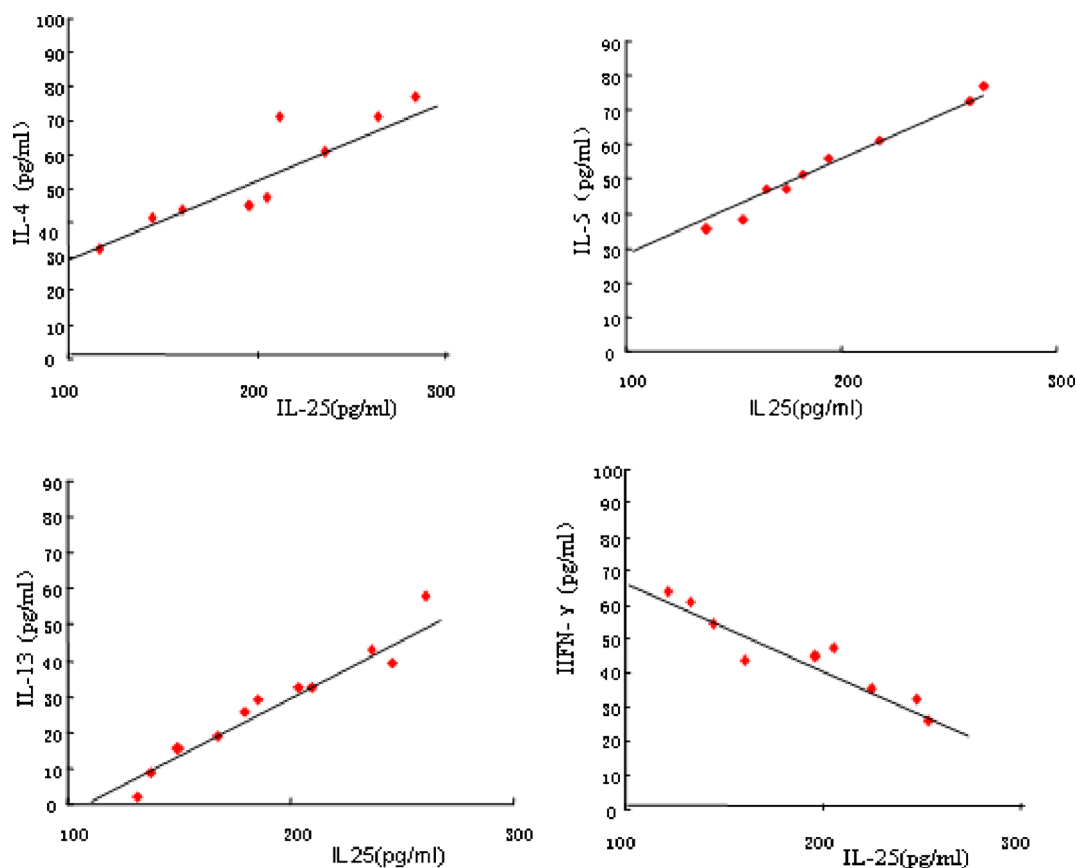


Fig. 4. Tight connection between the expression of IL-25 and IL-4, IL-5, IL-13 and IFN- γ in BALF. To clarify the effect of IL-25 in Th2 inflammatory responses in airway, we did a correlation analysis between expression of IL-25 and IL-4, IL-5, IL-13 and IFN- γ . ($r_{IL-4}=0.748$, $r_{IL-5}=0.691$, $r_{IL-13}=0.793$, $r_{IFN-\gamma}=-0.574$, $p<0.05$).

activate DCs population and interact with such a range of mediators to trigger the activation of Th2-type asthmatic cytokines network.

Inhaled Corticosteroids Inhibited Inflammatory Cytokines and Co-stimulatory Molecules Expression

To determine the critical role of corticosteroids in the development of asthma, inhaled budesonide was used during the phase of OVA challenge. Then the expression of eosinophils in airway and IL-25, IL-4, IL-5, IL-13 and IFN- γ as well as CD80 and CD86 in BALF were assessed. The mice treated with inhaled budesonide showed a decrease in eosinophils infiltration (Fig. 5a) and IL-25 mRNA expression in airway (Fig. 5c); the levels of IL-4, IL-5 and IL-13 in BALF were also decreased while the IFN- γ was increased in BALF in mice treated with inhaled budesonide compared with the OVA-treated mice (Fig. 5b). Moreover, the mice that received inhaled budesonide also showed a reduction in CD80 and CD86 expression on DCs in BALF (Fig. 5d). However, there was no difference between the budesonide-treated group and the control group.

DISCUSSION

Bronchial asthma is an airway inflammation accompanied by increased secretion of cytokines and cells. IL-25 was identified as being necessary for the up-regulation of the Th2 effector cytokines and mirroring the role of Th1 immunity response in asthma [1], but the interaction of IL-25 and Th2 inflammatory cytokines such as IL-4, IL-5, and IL-13 was unclear. Furthermore, the mechanisms underlying the regulation of IL-25 in the interaction of innate and adaptive immunity responses of asthma are relatively unexplored. In this study, we detected that the expression of IL-25 was tightly positive correlated with the levels of IL-4, IL-5 and IL-13 and negative correlated with the levels of IFN- γ , which demonstrated that IL-25 was emerging as a key regulator of asthmatic inflammation because of its ability to promote Th2, while suppressing Th1 cytokine responses. There are controversial reports regarding the cell types that produce IL-25. For example, Kim *et al* [3], showed that IL-25 was expressed exclusively by Th2 cells, whereas Wang *et al* [4], demonstrated that eosinophils and basophils, but not other immune cells (T cells, B cells, monocytes, dendritic cells, *etc.*), secreted IL-25. More recently, it was demonstrated that IL-25 mRNA was up-regulated in the gut after *Nippostrongylus brasiliensis*

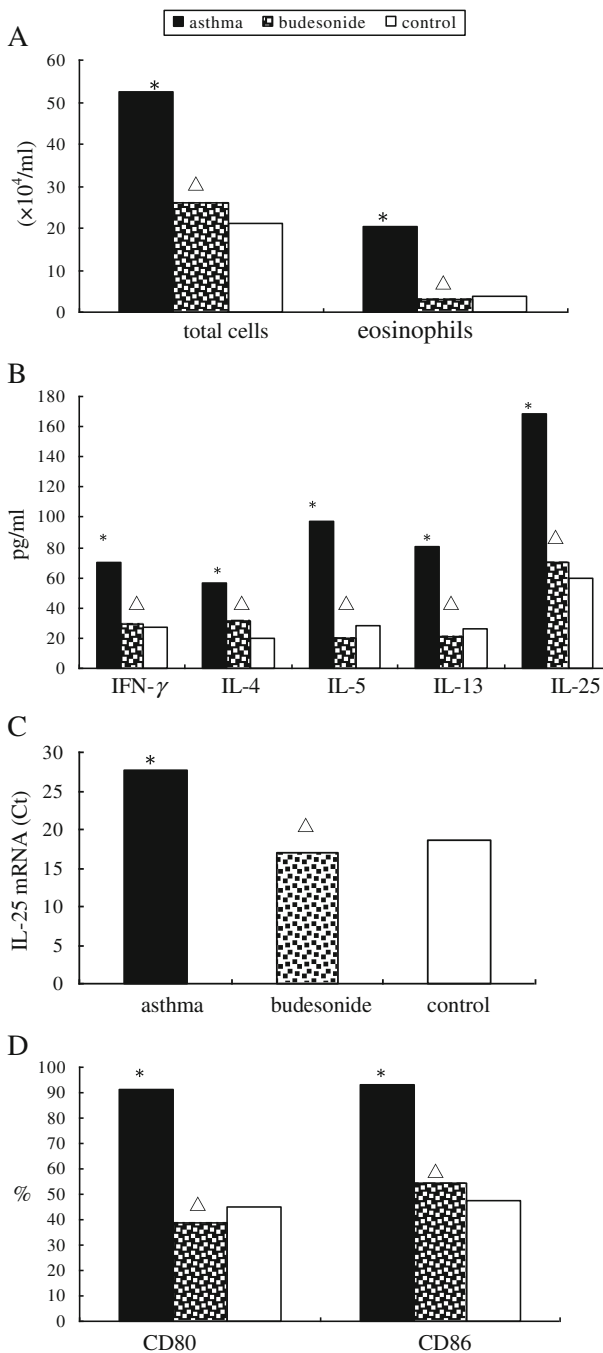


Fig. 5. The effect of budesonide inhalation in airway epithelium. **a** Eosinophils and total cell number in BALF. **b** The levels of IL-25, IL-4, IL-5, IL-13 and IFN- γ in BALF. **c** IL-25 mRNA expressed in airway epithelium. **d** Expression of CD80 and CD86 on DCs in BALF.

infection or in the lung in response to *Aspergillus fumigatus* infection [10]. Our immunohistochemistry analysis showed that IL-25 was highly expressed in epithelial cells

of the airway in mice with asthma. We also demonstrated that in the lungs of asthmatic mice, the expression of IL-25 mRNA and protein is increased in the airway epithelium. However, the principal source of IL-25 remains to be further identified.

DCs are professional antigen-presenting cells that bridge between innate and adaptive immunity [11]. In our study, IL-25 could activate DCs with high levels of co-stimulatory molecules CD80 and CD86 expression and interact with other inflammatory mediators to initiate the ensuing adaptive Th2-type immunity responses in asthmatic mice.

ICS treatment has long been considered a good approach to decrease asthmatic inflammation [12] in the clinic. In our study, when asthmatic animals were given ICS treatment, the airway inflammation could also be inhibited by the reduced infiltration of inflammatory cells and cytokines. The animals receiving budesonide inhalation also showed a significant decrease in the expression of CD80 and CD86 on DCs. As IL-25 release early in the allergic inflammatory cascade, budesonide inhalation might inhibit both the activation of DCs as well as Th2-related mediators produced [13]. However, other studies have found that some asthmatic patients were resistant to corticosteroids treatment even with high dose [14] and some animal studies have showed that Th17 cells and its IL-17 family members might explain the steroid-resistant asthmatic inflammation. Since IL-25 (IL-17E) is one of the IL-17 cytokine family members, IL-25 signaling pathway might provide a novel target for steroid-resistant asthmatic therapy, which needs further research into the mechanisms underlying asthma's pathophysiology. Although our data supported the idea that IL-25 interacted with other Th2 cytokines in asthmatic inflammation, other studies showed that for mice deficient in IL-4, IL-5, and IL-13 this could also induce AHR and the classic Th2 inflammatory responses with IL-25 is administration independent, which also warrants further research [15]. Further investigation is also needed to determine whether IL-25 acts through the induction of known broncho-constrictors such as the leukotrienes, or via molecules such as the recently described acidic chitinase.

In summary, the expression of IL-25 was increased in the airway epithelium of asthmatic inflammation and might be related to modulate DCs activation and Th2 immunity responses [16], while budesonide inhalation could probably mediate through the DC pathway to inhibit asthmatic airway inflammation [17] and might provide a novel target in the therapeutic intervention of asthma.

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